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Age-stratified reference intervals unlock the clinical potential of circulating cell-free DNA as a biomarker of poor outcome for healthy individuals and patients with colorectal cancer

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Abstract

Circulating cell-free DNA (cfDNA) has spurred much interest as a biomarker in oncology. However, inter- and intra-individual cfDNA levels vary greatly. Consequently, in order to base clinical decisions on cfDNA measurements, normal reference intervals are essential to avoid that ordinary variation is confused with clinically relevant change. The lack of reference intervals may potentially explain the ambiguous results reported in the field. Our study aimed to establish reference intervals and to evaluate the association between cfDNA and demographic and clinical variables, including colorectal cancer (CRC). Plasma samples and clinical data from 2817 subjects were collected including 1930 noncancer individuals and 887 CRC patients. cfDNA was measured using droplet digital polymerase chain reaction (PCR). The large cohort combined with robust cfDNA quantification enabled establishment of reference intervals (<67 years: 775-4860 copies/mL; ≥67 years: 807-6561 copies/mL). A cfDNA level above the age-stratified 90% percentile was prognostic of reduced survival in both noncancer individuals and CRC patients, with HR values of 2.56 and 2.01, respectively. Moreover, cfDNA levels increased significantly with age, elevated BMI and chronic diseases. In CRC, the cfDNA level was increased for Stage IV, but not Stage I to Stage III cancer. In summary, the use of reference intervals revealed that high cfDNA levels were predictive of shorter survival in both noncancer

Abbreviations: BMI, body-mass index; cfDNA, circulating cell-free DNA; CI, confidence interval; CRC, colorectal cancer; ctDNA, circulating tumor DNA; ddPCR, droplet digital PCR; HR, hazard ratio; IBD, inflammatory bowel disease; OS, overall survival; RA, rheumatoid arthritis.

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individuals and CRC patients, and that CRC development did not affect the cfDNA level until metastatic dissemination. Furthermore, cfDNA levels were impacted by age and chronic diseases. Conclusively, our study presents reference intervals that will help pave the way for clinical utilization of cfDNA.

KEYWORDS
biomarker, circulating cell-free DNA, colorectal cancer, overall survival, reference intervals

1 | INTRODUCTION

In the last decade, circulating cell-free DNA (cfDNA) has been extensively studied as a diagnostic, prognostic and predictive biomarker of somatic disease, especially in oncology.[1-5] However, many of the results have been conflicting and ambiguous, and possibly therefore the use of cfDNA has still not been implemented in clinical practice. In order to make clinical decisions based on biological measurements, it is essential to determine a reference interval so that clinically relevant, pathological changes are not confused with ordinary variation.

The cfDNA level is an equilibrium of cfDNA release/clearance and plasma volume. The major cell types contributing to the cfDNA pool are white blood cells, erythrocyte progenitors, endothelial cells and hepatocytes.[6,7] In metastatic cancer, the malignant cells may also provide a substantial contribution.[8] The cfDNA concentration may vary considerably between individuals[9,10] and moreover, intraindividual variance has been reported during the day[11,12] and with physical activity.[13,14] cfDNA levels have further been shown to increase significantly with age,[9,15,16] but whether this is due to the accumulation of age-related chronic diseases remains unknown. The establishment of cfDNA reference intervals in a robust cohort with a standardized analytical pipeline is thus necessary to forward cfDNA biomarker research and facilitate the implementation in daily clinical routine.

The present study aimed to establish reference intervals for cfDNA levels in healthy elderly individuals. It further investigated the association between cfDNA levels and age, demographic factors, chronic diseases and colorectal cancer (CRC) characteristics. Finally, it analyzed whether aberrantly high cfDNA levels were prognostic for decreased overall survival (OS) in both noncancer individuals and patients with CRC.

What's new?

Circulating cell-free DNA (cfDNA) has spurred much interest as a potential biomarker in oncology. However, large inter- and intra-individual variations have led to ambiguous study results and hampered the identification of clinically relevant changes. This study analyzes circulating cfDNA levels in a large cohort of 2817 individuals, with 887 colorectal cancer patients among them. The use of age-stratified cfDNA reference intervals reveals that aberrant cfDNA levels are prognostic of overall survival in both cancer and non-cancer individuals, independently of age, gender, and multimorbidity. Altogether, the results could facilitate the interpretation of cfDNA cancer research and clinical use of cfDNA biomarkers.

2 | MATERIALS AND METHODS

2.1 | Subjects

Research subjects were recruited in the period 2006 to 2019 from Danish surgical centers at Bispebjerg, Herlev, Herning, Hillerød, Horsens, Hvidovre, Randers, Aarhus and Viborg (flowchart of inclusion in Supplementary Figure 1). They were analyzed for cfDNA in various in-house studies,[2,17-21] and further included in the present study in a cross-sectional design. Subjects comprised both noncancer individuals and patients with CRC. The noncancer individuals were recruited through the population-based Danish National CRC Screening Program, offered to all Danes between 50 and 75 years of age, and from subjects referred to colonoscopy due to CRC-related bowel symptoms. Blood was collected after bowel preparation, but prior to colonoscopy. If the noncancer individuals did not undergo colonoscopy, blood was collected at the subject's convenience. The patients with CRC were recruited at diagnosis and blood was collected prior to operation. Inclusion criteria were age >18; exclusion criteria were acute illness, pregnancy and a diagnosis of cancer other than CRC <3 years before blood collection. Furthermore, all included CRC patients were treatment naïve, that is, patients treated with neo-adjuvant chemotherapy or radiation therapy prior to blood sampling were excluded. The study was conducted in accordance with the Helsinki Declaration. All subjects provided written informed consent. The use of biological material and clinical data was approved by the National Committee on Health Research Ethics and the Danish National Data Protection Agency.

2.2 | Clinical data collection

Data were collected from inclusion questionnaires, electronic patient files, the Danish Colorectal Cancer Group database[22] and the national Danish Personal Registry.[23] Diseases were grouped in categories. "Heart disease" included previous acute myocardial infarction, angina,

chronic ischemic heart disease, atrial fibrillation/flutter, valvular heart disease, heart failure and conductive disorders. "Diabetes" included both Type 1 and Type 2. "Inflammatory bowel disease" (IBD) included ulcerative colitis and Crohn's disease. "Pulmonary disease" included chronic asthma, chronic emphysema and chronic bronchitis. Also, diagnoses of hypertension and rheumatoid arthritis (RA) were registered.

2.3 | Isolation of cfDNA

In accordance with a prestudy defined standard operating procedure, whole blood was collected in BD Vacutainer K2 EDTA tubes (Becton Dickinson) and processed within 2 hours from venipuncture. The plasma was isolated by double centrifugation at 3000*g* for 10 minutes at 20°C, transferred to cryotubes (TTP) and stored at −80°C. Blood from CRC patients and noncancer individuals was processed identically. The plasma was transferred from local hospitals to a central freezer facility for storage until cfDNA extraction. cfDNA extraction was performed at a single facility: in brief, 4 to 8 mL of plasma was thawed at room temperature and cfDNA was extracted fusing a QIAsymphony robot (Qiagen) using the QIAsymphony DSP Circulating DNA kit (Cat No./ID: 937556, Qiagen) according to the manufacturer's instructions. Purified cfDNA was eluted in LoBind tubes or LoBind 96-well plates (Eppendorf AG) and stored at −80°C until analysis. The time from plasma storage to cfDNA quantification was recorded as "freeze time."

2.4 | Droplet digital PCR, quality control and cfDNA quantification

Droplet digital PCR (ddPCR) was used for cfDNA quantification and quality control of the cfDNA extraction procedure throughout the study. ddPCR was conducted in accordance with the dMIQE guidelines.[24] All ddPCR analyses were performed at the Department of Molecular Medicine, Aarhus University Hospital, using the standard operating procedure, defined prior to the initiation of the first study. The reaction mix, containing all reagents (18 pmol forward primer, 18 pmol reverse primer, 5 pmol probe, 2xSupermix for Probes no UTP [Bio-Rad]) except template DNA, was prepared in an isolated pre-PCR room to avoid contamination. Just before droplet generation, 2 μL template DNA was added to each reaction, bringing the final volume to 22 μL. One-nanoliter droplets were generated on the QX200 AutoDG Droplet Generator (Bio-Rad), using the procedure described by the manufacturer. After droplet generation, samples were amplified by PCR in an S1000 Thermal Cycler (Bio-Rad) at 95°C for 10 minutes and 45 cycles of 95°C for 30 seconds, 56°C for 1 minute and 98°C for 10 minutes and analyzed on a QX200 reader (Bio-Rad). All data was Poisson corrected. Positive and no-template controls were included for each assay and each batch.

To estimate cfDNA purification efficiency and assess the amount of DNA contamination from lysed lymphocytes in each sample, a duplex ddPCR assay was used as previously described.[21] In brief, a fixed amount of soybean CPP1 DNA fragments was added to each plasma sample before cfDNA extraction, and thereafter the purification efficiency was calculated as the percent recovery of CPP1 fragments. Lymphocyte DNA contamination from peripheral blood cell lysis was estimated by an assay targeting the VDJ rearranged IGH locus specific for B cells.[25] Samples with lymphocyte DNA contamination were excluded. The duplex assay details are given in Supplementary Table 1.

To estimate the cfDNA concentration, the number of cfDNA templates in plasma was quantified, using a ddPCR assay targeting a reference region on Chromosome 3. This region rarely shows copy number variation in CRC.[26] Primer and probe sequences for this assay are likewise provided in Supplementary Table 1.

2.5 | Statistical methods

Descriptive statistics for non-Gaussian distributed data were reported as medians with 10% and 90% percentiles, and for Gaussian distributions as mean with 95% confidence interval (CI). In linear regression analyses, non-Gaussian distributions were natural log transformed to achieve a log-Gaussian distribution and the statistical significance was assessed using *t*-tests. Of potential biases, bowel preparation has been found to have a minor systematic effect on the plasma cfDNA level[27] and freeze time, defined as the time from venipuncture until cfDNA extraction and quantification, has been suggested to decrease cfDNA concentration.[28] Furthermore, the cfDNA analyses were conducted in batches, in relation to in-house studies, in the period 2016 to 2019. Accordingly, these potential biases were adjusted for in all regression analyses.

Establishment of reference intervals for cfDNA was done a posteriori following guidelines from the Clinical and Laboratory Standards Institute.[29] The preanalytical conditions were evaluated, and the workable subject preparation as well as the strict regulations for specimen collection were standardized as previously described.[30] Reference intervals were made on log-Gaussian data. No outliers were identified with Dixon's method.[31] The reference limits were defined as the 2.5% and 97.5% percentiles and the 95% CI values of the upper/lower limits were calculated.

Follow-up time and OS were calculated from the time of blood collection to the time of death, other censoring or end of follow-up (1 March 2019). The associations between survival and independent variables were analyzed by Cox regressions. For all statistical analyses, a two-sided *P* value below .05 was considered significant. All assumptions for the statistical models were fulfilled. For statistical analyses, STATA V.12.1 (StataCorp LP, TX) was used.

3 | RESULTS

A total of 2820 subjects were included in the cohort: 1931 noncancer individuals without cancer at time of blood sampling, and 889 patients

with CRC. All CRC patients underwent colonoscopy as part of the diagnostic work-up. Of the noncancer individuals, 1056 underwent colonoscopy. Of these, 838 had a "clean colon" and 218 had adenomas. The remaining 875 noncancer individuals were all screening participants, who tested negative, and therefore did not undergo colonoscopy. Blood samples were collected from all individuals and the plasma cfDNA level determined by ddPCR. During experimental analysis, three samples were excluded (3/2820, 0.1%): two due to cfDNA measurement failure and one due to lymphocyte DNA contamination, leaving 1930 noncancer individuals and 887 patients with CRC for analyses. The median freeze time for a plasma sample was 1060 days, ranging from 66 to 3971 days. Freeze time of ≥5 years was associated with a decrease in cfDNA concentration and was thus included as a variable in all subsequent regression analyses (Supplementary Figure 2). The mean age was 66 years and 57.9% were males (Supplementary Table 2). The median follow-up time was 1457 days (437-4768 days). During follow-up, 259 individuals died (9.2%) and 5 were censored due to migration to other countries (0.2%). For 2409 of the included individuals, information about chronic diseases was available: 1419 had no diseases, and 990 had one or more diseases.

3.1 | Establishment of cfDNA reference intervals

The median cfDNA level of all 1930 noncancer individuals was 2051 copies/mL plasma and increased significantly with increasing age binned by decades (Figure 1A). A reference sample group was defined, consisting of the subset of noncancer individuals without chronic disease, who did not undergo colonoscopy. The latter because the

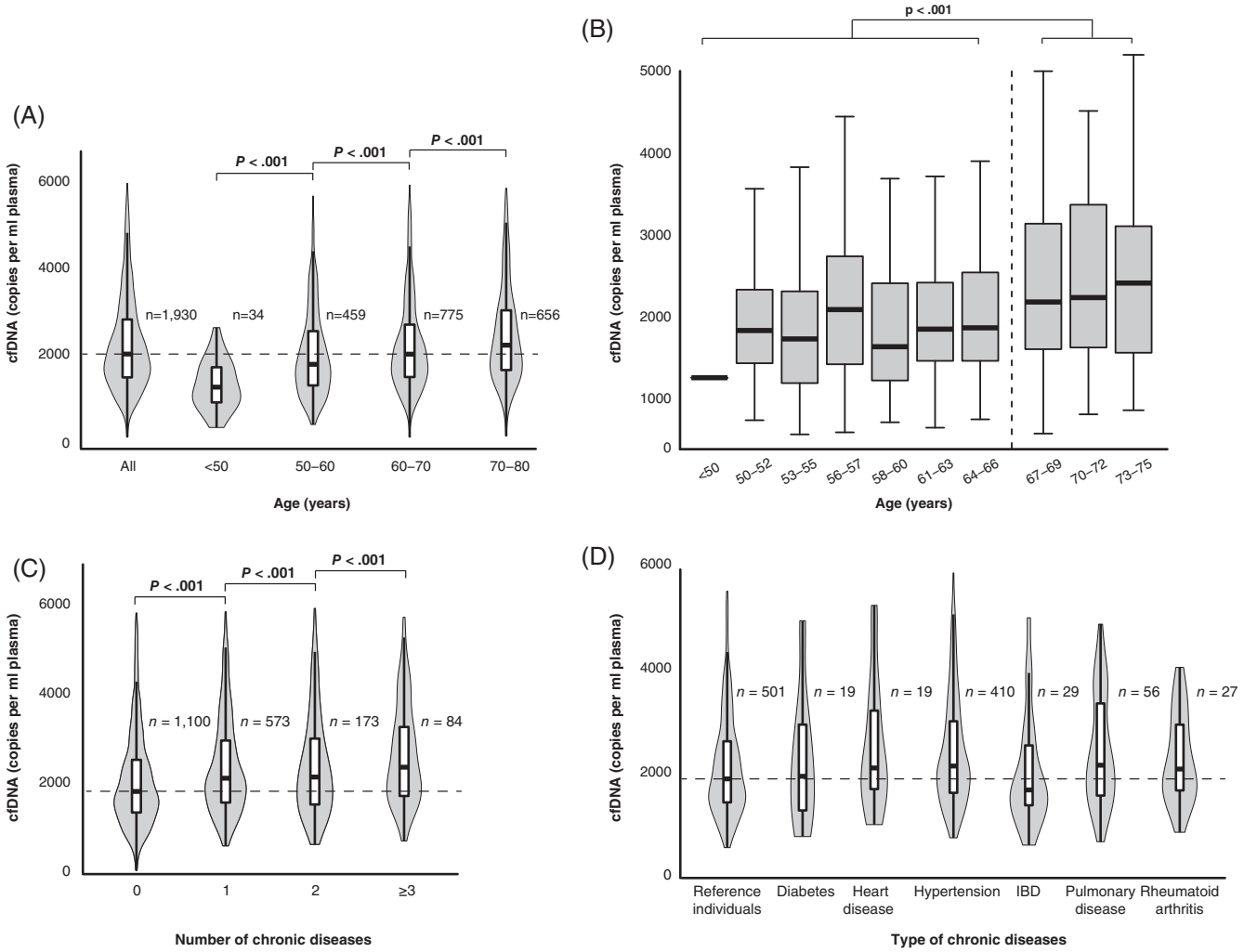

FIGURE 1 cfDNA levels in noncancer individuals. A, Violin plots showing the distribution of cfDNA levels, stratified by 10-year intervals, in all individuals. B, Box plots illustrating the distribution of cfDNA levels in all reference individuals in 3-year intervals. Between "64-66" and "67-69" years, the median increased, and therefore 67 was chosen as cut-off for the age-dependent substratification of reference intervals. C, Violin plots showing cfDNA levels for noncancer individuals stratified by chronic diseases number. Age stratified values presented in Table 2. D, Violin plots showing cfDNA levels in noncancer individuals by disease group. IBD, inflammatory bowel disease. Box plot shows the median (line) and the interquartile range (box), while violins represent the distribution of cfDNA. Box plot whiskers represent the minimum and maximum values. Differences in cfDNA levels were tested with *t*-test, *P* value <.05 considered statistically significant

colonoscopy bowel preparation was shown to cause a minor (7.7%) increase in the cfDNA level (Supplementary Table 3). The reference sample group consisted of 501 individuals ("reference individuals") and to estimate the impact of age on cfDNA levels in the reference individuals, the cfDNA levels were displayed in small intervals (Figure 1B). An increase in the median cfDNA level was revealed at age 67 and above, indicating a need for age-stratified reference intervals. Accordingly, the reference group was divided into two subgroups: below 67 years and 67 years or above. The age-stratified cfDNA levels in the two groups were significantly different (median 1839 and 2252 copies/mL, respectively, $P < .001$ in t-test) and the fraction of individuals in each group that were outside the total reference interval (2.5% under 792 copies per mL for age < 67 years and 4.8% over 5477 copies per mL for age ≥ 67) was above the 4.1%, at which it is recommended to partition the reference group.[32] Consequently, age-stratified reference intervals were established for individuals <67 and ≥67 years (Table 1). These were 775 to 4860 copies/mL and 807 to 6561 copies/mL, respectively.

3.2 | Associations between cfDNA and demographic variables and chronic diseases

Elevated cfDNA levels were significantly associated with age and increasing BMI ($P < .001$), while there was a tendency toward an association between increased cfDNA levels and male gender ($P = .082$). Smoking was associated with significantly decreased cfDNA levels (Supplementary Table 4). An association between cfDNA levels and alcohol consumption could not be shown. When compared to the reference individuals, the cfDNA levels in individuals with chronic disease were significantly elevated. Stratification for number (Figure 1C) and type of chronic disease (Figure 1D) revealed a significantly increasing cfDNA level with an accumulating number of chronic diseases and intertype variation. Univariate regression analyses confirmed these findings (Supplementary Table 4).

All significant variables were combined in a multivariable regression analysis (Table 2). Increased age and BMI remained significantly associated with higher cfDNA levels, $P < .001$. Likewise, the association between smoking and reduced cfDNA levels remained. Oppositely, the weaker association with male gender disappeared completely. For chronic diseases, "one disease" and "three or more diseases" remained significantly associated with higher cfDNA levels.

3.3 | Associations between cfDNA and tumor characteristics in patients with CRC

To investigate if characteristics of adenomas and CRCs were associated with increased cfDNA levels, data from 887 CRC patients and 218 individuals with adenomas were analyzed (Figure 2, Table 3). Reference individuals and individuals with adenomas had comparable cfDNA levels. For CRC patients, the cfDNA level was significantly higher, but when stratified for stage and age, the difference was found to be driven by Stage IV patients (Table 3). The cfDNA levels in plasma from patients with Stages I to III were similar to that of reference individuals. The association between cfDNA and other

TABLE 2 Multivariable regression of the association between cfDNA levels and age for noncancer individuals

Multivariable linear regression	Regression coefficient[a]	*P* value
Age (continuous)	0.01	<.001
Gender		
Female	—	—
Male	0.004	.862
BMI (continuous)	0.03	<.001
Smoking		
Nonsmoker	—	—
Smoker	−0.13	<.001
Former smoker[b]	−0.01	.706
Number of chronic diseases		
No disease	—	—
One disease	0.08	.006
Two diseases	0.03	.443
Three or more diseases	0.14	.023

[a]Data log transformed to achieve Gaussian distribution. Adjusted for bowel preparation and freeze time.
[b]Former smoker: nonsmoker for >6 months; cfDNA: circulating cell-free DNA, BMI: bodymass index.

TABLE 1 cfDNA reference intervals stratified by age

	n	Reference interval, cfDNA copies/mL plasma[a]	Lower limit 95% CI, cfDNA copies/mL plasma	Upper limit 95% CI, cfDNA copies/mL plasma	*P* value	cfDNA copies/mL plasma median (10%;90%)
All individuals	501	792 to 5477	707 to 886	4837 to 6620		1971 (1122;3712)
By age						
<67	317	775 to 4860	693 to 883	4070 to 6552	—	1839 (1047;3506)
≥67	184	807 to 6561	608 to 985	5279 to 10406	<.001	2252 (1294;3943)

Abbreviations: CI, confidence interval; n, number; cfDNA, circulating cell-free DNA; 10%;90%, percentiles.
[a]According to Reference 32, subgroups should be made if >4,1% of the values lie outside the upper limit of the common reference interval. 4.8% of the group ≥67 years had values outside this. Cut-off age estimated from Figure 1B. Difference in cfDNA level between groups estimated by using t-test.

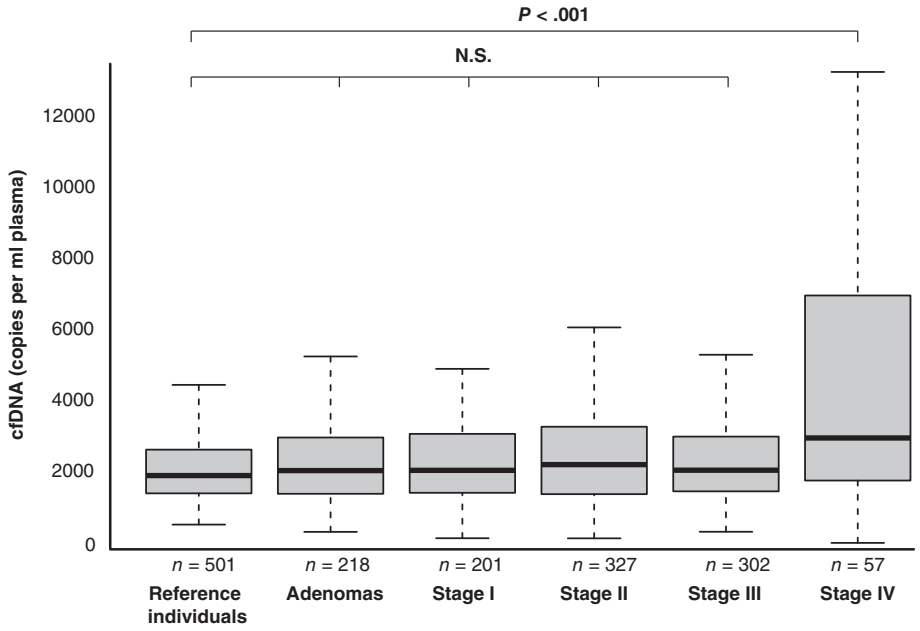

FIGURE 2 cfDNA levels during CRC development. Box plot showing the cfDNA levels through CRC development in reference individuals, adenomas and CRC stages I to IV. Box plot illustrates the median (line) and the interquartile range (box). Whiskers represent the minimum and maximum values. Differences in cfDNA levels tested with *t*-test, *P* value <.05 considered statistically significant. CRC, colorectal cancer; N.S., nonsignificant

TABLE 3 Univariable associations between cfDNA levels, adenomas and CRC characteristics

Univariable linear regression	n (%)	cfDNA copies per mL plasma[a] median (10%;90%)	Regression coefficient	*P* value
Adenomas and CRC				
Reference individuals	501	1971 (1122;3712)	—	—
Adenomas	218	2113 (1080;4240)	0.06	.291
All CRC stages	887 (100)	2185 (1024;5750)	0.12	.015
Age (continuous)[b]			0.01	<.001
CRC stage				
Reference individuals			—	—
Stage I	201 (22.7)	2120 (1033;4537)	0.09	.137
Stage II	327 (36.9)	2282 (990;5096)	0.09	.179
Stage III	302 (34.1)	2123 (1005;5457)	0.08	.207
Stage IV	57 (6.4)	3036 (1336; 31592)	0.78	<.001
Test for trend, cfDNA increase across stages				<.001
Tumor (pT stage)				
T1	124 (14.0)	2066 (932;4826)	—	—
T2	103 (11.6)	2211 (1140;4746)	0.03	.741
T3	494 (56.0)	2128 (948;4950)	−0.03	.697
T4	140 (15.8)	2339 (1116;7618)	0.16	.098
Missing	26 (2.9)			
Lymph node (pN stage)				
N0	371 (41.8)	2153 (983;5002)	—	—
N1	198 (22.3)	2217 (924;6089)	0.03	.621
N2	126 (14.2)	2058 (1037;6135)	−0.01	.998
Missing	192 (21.6)			
Metastasis (pM stage)				
M0	830 (93.6)	2158 (1011;5049)	—	—
M1	57 (6.4)	3036 (1336;31 592)	0.67	<.001

TABLE 3 (Continued)

Univariable linear regression	n (%)	cfDNA copies per mL plasma[a] median (10%;90%)	Regression coefficient	*P* value
Tumor location[b]				
Anus and rectum	181 (20.4)	2125 (902;4281)	—	—
Left colon[c]	361 (40.7)	2185 (967;5897)	0.09	.210
Right colon[d]	325 (36.6)	2244 (1150;6162)	0.10	.162
Missing	20 (2.3)			
Histological type[b]				
AC	624 (70.4)	2304 (1130;5750)	—	—
Mucinous AC	43 (4.9)	2260 (1278;8168)	0.15	.150
Neuroendocrine AC	5 (0.6)	—	—	—
Signet ring cell AC	3 (0.3)	—	—	—
Missing	212 (23.9)			
Tumor differentiation[b]				
High	3 (0.3)	1757 (1358;2458)	—	—
Medium	318 (35.9)	2396 (1114;5997)	0.22	.606
Low	73 (8.2)	1996 (1190;8002)	0.27	.530
Missing	493 (55.6)			
Tumor diameter mm[b]				
<25	39 (4.4)	1980 (1202;8002)		
25 to 50	221 (24.8)	2185 (878;6270)		
50 to 75	168 (18.9)	2283 (1114;4950)		
75 to 100	64 (7.2)	2437 (1084;8688)		
>100	36 (4.1)	2654 (1358;7728)		
Missing	360 (40.6)			
Tumor size (continuous)			0.03	.028
Perineural growth[b]				
No	281 (31.7)	2359 (1089;6510)	—	—
Yes	160 (18.0)	2287 (1195;6817)	−0.05	.511
Missing	446 (50.3)			
Satellites[b]				
No	370 (41.7)	2354 (1207;6603)	—	—
Yes	60 (6.8)	2491 (1359;7700)	0.04	.719
Missing	457 (51.5)			
Venous invasion[b]				
No	320 (36.1)	2234 (1153;5730)	—	—
Yes	135 (15.2)	2557 (1176;8893)	0.16	.066
Missing	432 (48.7)			
Tumor budding[b]				
No	345 (38.9)	2350 (1217;6837)	—	—
Yes	68 (7.7)	2605 (1105;7508)	0.07	.523
Missing	474 (53.4)			

Abbreviations: AC, adenocarcinoma; cfDNA, circulating cell-free DNA; CRC, colorectal cancer; n, number; %, percentage; 10%;90%, percentiles.

[a]Data log transformed to achieve Gaussian distribution. All linear regressions adjusted for study origin and freeze time.

[b]Adjusted for CRC stage.

[c]Sigmoid, descending, splenic flexure.

[d]Transverse, hepatic flexure, ascending, coecum.

TABLE 4 Associations between cfDNA levels and OS

	Noncancer individuals			Patients with CRC		
Multivariable Cox regression, survival analysis	HR	95% CI	*P* value	HR	95% CI	*P* value
Aberrant cfDNA level[a]						
No	—	—	—	—	—	—
Yes	2.56	1.37 to 4.78	.003	2.01	1.17 to 3.43	.011
Age	1.13	1.09 to 1.17	<.001	1.05	1.03 to 1.08	<.001
Male gender	1.45	0.83 to 2.50	.182	0.98	0.61 to 1.60	.942
Smoking						
Nonsmoker	—	—	—	—	—	—
Smoker	2.41	1.33 to 4.37	.004	1.66	0.92 to 3.00	.090
Former smoker[b]	1.56	0.78 to 3.09	.207	1.24	0.75 to 2.04	.401
BMI (continuous)	0.94	0.88 to 1.01	.095	0.96	0.92 to 1.00	.051
Alcohol consumption						
0 units/week	1.44	0.69 to 2.97	.345	1.24	0.73 to 2.10	.427
1 to 7 units/week	—	—	—	—	—	—
8 to 14 units/week	0.74	0.36 to 1.53	.413	0.83	0.41 to 1.69	.614
15 to 21 units/week	0.80	0.32 to 1.97	.622	1.93	0.93 to 3.98	.074
>21 units/week	2.23	1.00 to 5.00	.051	1.14	0.49 to 2.61	.765
Number of chronic diseases						
No disease	—	—	—	—	—	—
One disease	1.67	0.86 to 3.23	.130	0.89	0.42 to 1.86	.744
Two diseases	1.53	0.60 to 3.92	.395	1.74	0.82 to 3.72	.151
Three or more diseases	2.87	1.11 to 7.41	.029	1.44	0.31 to 6.63	.636
CRC stage						
Stage I	—	—	—	—	—	—
Stage II	—	—	—	0.87	0.42 to 1.82	.727
Stage III	—	—	—	2.13	1.03 to 4.39	.040
Stage IV	—	—	—	35.0	17.2 to 71.2	<.001

[a]Aberrant cfDNA limit: >90% percentile for age-stratified cfDNA reference level (Table 1). Adjusted for bowel preparation, study origin and freeze time.
[b]Former smoker defined as nonsmoker >6 months; BMI, bodymass index; CI, confidence interval; cfDNA, circulating cell-free DNA; CRC, colorectal cancer; HR, hazard ratio.

histopathological characteristics was also evaluated: cfDNA levels increased with increasing tumor size (P = .028), but no other significant associations were found. cfDNA levels for CRC patients with comorbidities are shown in Supplementary Table 5.

3.4 | Associations between cfDNA and OS

To evaluate if high cfDNA levels were associated with decreased OS, an "aberrant cfDNA limit" was defined as the age-stratified 90% percentile for reference individuals (Table 1). Using this cut-off, the noncancer individuals and patients with CRC were separated into two groups: normal or aberrant cfDNA level (below or above the limit, respectively). Cox regression analyses were used to assess the association between the two groups and OS. The analyses were adjusted for variables expected to influence OS or cfDNA levels, that is, age, gender, BMI, smoking status, alcohol consumption, chronic diseases and CRC stage (Table 4). For both noncancer individuals and CRC patients, aberrantly high cfDNA levels were significantly associated with decreased OS (HR 2.56 and 2.01, respectively). In noncancer individuals, the following variables were also associated with decreased OS: higher age, smoking and ≥3 chronic diseases. Alcohol consumption >21 units/week was borderline associated with a decreased OS (P = .051). In CRC patients, high age and Stages III and IV disease were associated with decreased OS. A trend toward shorter OS was also observed for smoking. Furthermore, a higher BMI was borderline associated with longer OS (P = .051).

4 | DISCUSSION

Clinical benefit of cfDNA as a cancer biomarker has not yet been documented, possibly due to the lack of standardized preanalytical conditions and relevant reference intervals. In our study, age-stratified

reference intervals for cfDNA levels in a population of healthy individuals were defined. The results showed that higher age, chronic diseases, and disseminated CRC were independently associated with increased cfDNA levels. Furthermore, the increase in cfDNA levels for several clinically important chronic diseases was quantified, and it was shown that aberrantly high cfDNA levels were an independent prognostic marker for shorter OS in both noncancer individuals and CRC patients.

To establish cfDNA reference values, a group of 501 healthy individuals, comprising both genders, were chosen from a population aged 50 to 80 years. Previously, it has been common practise to use, for example, healthy young males. However, such a reference group is irrelevant in most oncological settings, where the population is commonly above 50 years and of both sexes. The cfDNA measurement here was made without extensive subject preparation criteria (eg, time of day for blood collection or physical activity restriction, all of which have been shown to affect the cfDNA level[11-14]) and consequently the cfDNA levels may vary more than that if strict preanalytical criteria had been applied. However, this mirrors the clinical reality well, and when the results were controlled for potential outliers, the variance of the reference values was within the recommended limits.[29] Although reference intervals are often stratified by gender, it was not done here. The present data showed no difference between genders after adjusting for smoking status, alcohol consumption and chronic diseases in the multivariable regression analysis (Table 2).

Instead, the reference intervals were stratified by age. In agreement with previous publications,[9,15,16] the present data showed that cfDNA levels increased with higher age for both noncancer individuals (Table 2) and also for patients with CRC (Table 3). Furthermore, stratifications indicated that the association was neither confounded by gender, BMI, smoking or chronic disease for noncancer individuals nor by tumor stage for patients with CRC. Why cfDNA levels increase with age is not completely understood. Older age has previously been shown to be related to increasing inflammation, so-called "inflammaging."[33] As white blood cells, contributing greatly to the cfDNA pool, are involved in inflammaging, their increased turnover may be part of the explanation. In contrast to the present study, a recent meta-analysis of 892 healthy controls, pooled from eight different studies, found no significant association between cfDNA levels and age.[10] However, the included studies used different analytical methodologies for cfDNA quantification, which complicate the integration and interpretation of the result.[28] As recommended in recently published guidelines for cfDNA analysis,[34] the cfDNA levels reported in the present study were produced by absolute quantification by ddPCR.

Several small-scale studies have shown an association between acute diseases and increased cfDNA levels.[35-39] The present study showed that chronic diseases, independently of age, were associated with higher cfDNA levels as well. Although cfDNA levels are only transiently affected by acute disease, they may be permanently affected by chronic disease. The results further indicated that the effect of chronic diseases on the cfDNA level was cumulative, as the level increased with an increasing number of diseases. This suggests that cfDNA from all pathologically affected tissues accumulates in the blood. The association between cfDNA levels and, for example, cardiovascular disease could be confounded by gender, smoking status or alcohol consumption. Likewise, the association with, for example, diabetes could be confounded by higher BMI, which increases inflammation and cfDNA levels.[40,41] Owing to the large cohort size, it was possible to adjust for this in a multivariable regression: multimorbidity remained significantly associated with higher cfDNA levels. Moreover, the results showed that smoking was associated with decreased cfDNA levels. This association has not been described previously, and the underlying mechanism is unclear. No significant association between alcohol consumption and cfDNA levels was observed in the present data, though excessive alcohol intake may cause liver cell damage. These unexpected results could relate to the fact that the smoke/alcohol variables were self-reported, and such data can be heavily biased by the lack of memory or by misreporting due to fear of stigmatization (social desirability bias).

Previous research has shown that cancer cells contribute to the cfDNA pool, especially in disseminated disease,[8] and that cancer patients may have higher cfDNA levels compared to healthy individuals.[10] The present study included patients with CRC in all stages, as well as patients with adenomas. Comparable levels of cfDNA were observed in healthy individuals and individuals with adenomas, which is in agreement with findings from previous studies.[15,42] Furthermore, comparable cfDNA levels in healthy individuals and Stages I to III CRC were found; only patients with disseminated disease (Stage IV) had significantly increased cfDNA levels. This finding was supported by other previous reports.[9,43] Tumor characteristics associated with high cfDNA levels also included large tumor size. It has previously been shown that increased circulating tumor DNA (ctDNA) levels were associated with a tumor size above 5 cm.[43] This corroborates the present findings, as ctDNA is part of the total cfDNA quantity. Taken together, these findings indicate that CRC generally does not affect the overall cfDNA level until late in development.

To determine whether increased cfDNA levels were prognostic of OS, an "aberrant cfDNA limit" was defined prior to data analysis. The analysis revealed that aberrant cfDNA levels were associated with decreased OS independently of age, smoking, alcohol consumption, chronic diseases and CRC stage (Table 4). The significance of the result was emphasized as aberrant cfDNA levels were prognostic in both noncancer individuals and CRC patients. The association between elevated cfDNA levels and OS in CRC has been reported in previous studies including disseminated CRC[44] and CRC in all stages.[42,45] For noncancer individuals, increased cfDNA levels have been reported to be associated with decreased OS in individuals with acute disease.[39,46] Furthermore, it has been suggested that increasing cfDNA levels are a proxy marker for frailty in elderly.[47,48] Therefore, we hypothesize that aberrant cfDNA levels may be used as a proxy marker for overall health (regardless of CRC diagnosis) and that cfDNA levels possibly could be integrated in artificial intelligence algorithms, predicting patient outcome in future personalized oncological strategies. Whether the 90% percentile is the best clinical decision limits for the "aberrant cfDNA level" must be warranted in future studies.

The present study has some limitations. Firstly, though the Danish registries and databases are highly accurate and valid,[22,23] subjects registered as healthy may still have nondiagnosed chronic diseases. The risk of acute disease during blood collection is however not an issue, as acutely ill subjects were not included in the study. Secondly, even though subjects with a cancer diagnosis <3 years from blood collection were excluded, some of the participants may have occult recurrence of a former cancer or new, undiagnosed metachronous cancer. Thirdly, information on the chronic diseases treatment in the study group was not available. Hypothetically, well-regulated RA or IBD would cause less inflammation compared to dysregulated RA/IBD, which would impact the release of cfDNA from white blood cells. Overall, these limitations might increase the cfDNA reference intervals variance. However, we find this acceptable, as it reflects the clinical reality.

Conclusively, as numerous factors clearly affect cfDNA levels, cfDNA research results should be carefully interpreted. Our results underline that especially higher age and the presence of chronic diseases are associated with increased cfDNA levels, which could influence cfDNA research interpretation. For example, when results are presented as varying allele frequencies (ctDNA in the numerator, overall cfDNA level in the denominator), then interindividual comparison would be greatly influenced by, for example, differences in age. Another important finding is that aberrant cfDNA levels are prognostic for decreased OS for both noncancer and CRC individuals. Finally, by reporting reference intervals for cfDNA levels, we hope to unlock the clinical potential of cfDNA as a biomarker, and we foresee that cfDNA could be incorporated into future predictive oncological stratification tools and prognostic artificial intelligence algorithms.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was conducted in accordance with the Helsinki Declaration. All subjects provided written informed consent. The use of biological material and clinical data was approved by the National Committee on Health Research Ethics and the Danish National Data Protection Agency.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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